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The majority of our present chemotherapeutic agents only kill cells effectively when they are proliferating, this may explain why these agents have been of such limited success in patients. In contrast to these ineffective agents, we have chemically modified a plant toxin, Thapsigargin (TG), to produce primary amine-containing analogs that are potent, cell proliferation independent, inducers of apoptosis in prostate cancer cells. These TG-analogs, however, are not prostate cancer-specific cytotoxins. The original hypothesis was that these TG analogs could be converted to inactive prodrugs that can be efficiently converted back to active killing drugs only by the enzymatic activity of Prostate Specific Membrane Antigen (PSMA). Since PSMA is expressed in high levels only by prostate cancer cells and not by normal cells, this should allow specific targeting of the TG-analog's killing ability to prostate cancer cells. The objectives of the study were to first define a peptide substrate that was specifically hydrolyzed by PSMA. The second objective was to identify a cytotoxic TG analog that is inactivated upon coupling to this peptide. To accomplish the first objective, a larger number of peptide substrates for PSMA activity and specificity had to be rapidly screened. We began by synthesizing a series of analogs based on methotrexate consisting of the pteridine ring-para-aminobenzoic acid (APA) portion of methotrexate coupled to a variety of peptides. Using this approach a series of methotrexate analogs consisting of the APA moiety coupled to peptide substrates were screened for PSMA hydrolysis and serum stability. The preferred substrate had the sequence APA-Asp-Glu*Glu*Asp-Glu (where hyphen equals alpha linkage and star equals gamma). Cytotoxic glutamate and aspartate containing analogs of TG that can be coupled to this peptide carrier were also identified.

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Introduction:

The majority of our present chemotherapeutic agents only kill cells effectively when they are proliferating; this may explain why these agents have been of such limited success in patients. In contrast to these ineffective agents, we have chemically modified a plant toxin, Thapsigargin (TG), to produce primary amine-containing analogs that are potent, cell proliferation independent, inducers of apoptosis in prostate cancer cells. These TG-analogs, however, are not prostate cancer-specific cytotoxins. The original hypothesis was that these TG analogs could be converted to inactive prodrugs by coupling to a peptide carrier that is a substrate for Prostate Specific Membrane Antigen (PSMA). In this way the inactive prodrugs can be efficiently converted back to active killing drugs only by the enzymatic activity of PSMA. Since PSMA is expressed in high levels only by prostate cancer cells and not by normal cells, this should allow specific targeting of the TG-analog's killing ability to prostate cancer cells. The objectives of the study were to first define a peptide substrate that was specifically hydrolyzed by PSMA. The second objective was to identify a cytotoxic TG analog that is inactivated upon coupling to this peptide. Originally we had planned to couple the TG analog to a series of peptide sequences to assay PSMA specific hydrolysis. Due to the structure and highly lipophilic nature of the TG analog, we realized that using this approach for rapid screening of peptide substrates would be difficult and time-consuming. To rapidly screen a large number of peptide substrates an alternative approach was adopted. We began by synthesizing a series of analogs based on methotrexate consisting of the pteridine ring-para-aminobenzoic acid (APA) portion of methotrexate coupled to a variety of peptides, figure 1. This strategy was utilized for several reasons. The coupling of APA to the N-terminal amine of gamma-linked polyglutamates does not inhibit sequential PSMA hydrolysis. The chemistry to produce these analogs has already been described and the large quantities of the inexpensive APA precursor are available. The APA molecule has an absorbance at 340 nm and hydrolysis of prodrugs can be readily followed by HPLC analysis. This approach made possible the following accomplishments over the funding period: (1) synthesized and screened alpha-linked dipeptide analogs of methotrexate for PSMA specific hydrolysis; (2) synthesized and characterized a series of gamma-linked analogs di and tripeptide analogs of methotrexate for PSMA hydrolysis and hydrolysis by gamma glutamyl hydrolase; (3) synthesized and characterized a series of longer chain gamma-linked analogs of methotrexate and characterized PSMA hydrolysis, cytotoxicity, and stability in conditioned media and human and mouse plasma. (4) synthesized and characterized a series of methotrexate analogs with both alpha and gamma-linked amino acids and characterized PSMA hydrolysis, cytotoxicity, and stability in conditioned media and human and mouse plasma. In addition, we have synthesized and screened a series of TG analogs consisting of hydrocarbon linker coupled to amino acids and from a series of analogs we identified a gamma-glutamyl 12-aminododecanoyl TG analog that can be coupled to PSMA specific peptide substrate. In future studies TG prodrugs consisting of gamma and alpha-linked acidic amino acids can be synthesized, assayed for PSMA hydrolysis and for non-specific and specific cytotoxicity against PSMA-positive and negative prostate cancer cell lines. The relevance of this work to prostate cancer research is that TG, an effective prostate cancer cell cytotoxin, can be targeted to sites of metastatic prostate cancer without significant toxicity.

BODY:

PSMA is a 100 kDa prostate epithelial cell type II transmembrane glycoprotein that was originally isolated from a cDNA library from the androgen responsive LNCaP human prostate cancer cell line (Horoszewicz). Using a series of monoclonal antibodies, several groups have characterized PSMA expression in various normal and tumor tissues. These studies demonstrated that PSMA is expressed in high levels by both normal and malignant prostate tissue. Low-level expression was only seen in the lumen of the small intestine and the proximal tubules of the kidney. In addition, while normal vascular endothelial cells are PSMA negative, endothelial cells of the tumor vasculature stain positive for PSMA in a large number of tumor types, while the tumor cells themselves do not express PSMA.

In summary, the aforementioned studies highlight the characteristics of PSMA that make it a suitable target for prostate specific therapy. PSMA expression is highly restricted to prostate tissue with strongest expression in both primary and metastatic prostate cancers. The PSMA protein detectable in prostate cancers is an integral membrane protein and therefore has an extracellular domain that is accessible to agents in the extracellular peritumoral fluid making it possible to target this protein with antibodies and prodrugs. A final interesting aspect of PSMA expression is that the PSMA mRNA is upregulated upon androgen withdrawal. In LNCaP cells androgen has been found to downregulate PSMA expression and in patient specimens an increase in immunohistochemically detectable PSMA expression has been observed following androgen ablative therapy. In contrast, PSA expression is downregulated by androgen deprivation. Therefore, PSMA should be readily targetable in the majority of hormone refractory patients because PSMA levels are expected to remain high following androgen ablation.

Two discrete enzymatic functions for PSMA have been described. Initially, Carter et al demonstrated that PSMA possesses the hydrolytic properties of an N-acetylated α-linked acidic dipeptidase (NAALADase). NAALADase is a membrane hydrolase activity that is able to hydrolyze the neuropeptide N-acetyl-*l*-aspartyl-*l*-glutamate (NAAG) to yield the neurotransmitter glutamate and N-acetyl-aspartate. In addition to the NAALADase activity, PSMA also functions as a pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase). PSMA exhibits exopeptidase activity and is able to progressively hydrolyze γ-glutamyl linkages of both poly-γ-glutamated folates and methotrexate analogs with varying length glutamate chains. The role that PSMA's folate hydrolase activity plays in the physiology of prostate cells is presently unknown. PSMA has more recently been classified as glutamate carboxypeptidase II. Homologous proteins have been isolated from the rat brain and pig jejunum. These proteins have > 80% amino acid sequence homology with PSMA. Recently, it has been demonstrated that both the pig jejunal folylpoly-γ-glutamate carboxypeptidase and the rat brain NAALADase can also hydrolyze both poly-γ-glutamated folates as well as NAAG.

A variety of strategies can be used to target PSMA. In our original proposal, we outlined an approach that would take advantage of both the prostate specific expression of the PSMA protein in men and its unique NAALADase activity. In our original proposal, inactive prodrugs consisting of a potent cytotoxic analog of thapsigargin coupled to a peptide carrier can be designed such that the active thapsigargin analog is liberated only by the NAALADase activity of PSMA present on normal and malignant prostate epithelial cells. These prodrugs can be used to target PSMA-positive prostate cancer and could potentially be used to target the vascular endothelial cells of a wide variety of epithelial cancers.

A successful PSMA activated prodrug must pass three critical tests. The prodrug must be cleaved by PSMA, it must not be toxic to PSMA-negative cells and its toxicity to PSMA positive cells must be secondary to activation specifically by PSMA. In order to rapidly screen a larger number of prodrugs for PSMA activity and specificity, we began by synthesizing a series of analogs based on methotrexate consisting of the pteridine ring-para-aminobenzoic acid (APA) portion of methotrexate coupled to a variety of peptides, figure 1. This strategy was utilized for several reasons. The coupling of APA to the N-terminal amine of gamma-linked polyglutamates does not inhibit sequential PSMA hydrolysis. The chemistry to produce these analogs has already been described and the large quantities of the inexpensive APA precursor are available. The APA molecule has an absorbance at 340 nm and hydrolysis of prodrugs can be readily followed by HPLC analysis.

Finally, APA-Glu (i.e. methotrexate) is cytotoxic at nanomolar concentrations and therefore activation of prodrugs in vitro can be easily assessed using growth inhibition and clonogenic survival assays. This strategy, therefore, was used to identify a PSMA-specific peptide carrier. Once such a carrier is identified, the thapsigargin (TG) analog such as the aminophenylproprionyl derivative APT (See TG section for description) can then be substituted for the APA molecule and coupled to the peptide carrier.

The first step in this prodrug development strategy was to obtain enzymatically active, purified PSMA to use to screen potential substrates. A fusion protein consisting of the extracellular portion of the PSMA protein fused to an Fc fragment had been previously described. This protein has the transmembrane segment of the PSMA protein deleted to create a secreted fusion protein. This PSMA protein was provided to our laboratory by Lexigen Pharmaceuticals. Purified Fc-PSMA was found to have a specific NAALADase activity of 85.6 ± 5 nmol/min/mg protein. In comparison, PSMA specific NAALADase activity from LNCaP cell membrane preparations was 29.0 ± 2.6 pmol/min/mg protein. Similar to the wild type PSMA, the Fc-PSMA also functioned as an exopeptidase with pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase) activity as demonstrated by its ability to progressively hydrolyze γ-glutamyl linkages of MTX-Glu_4. Recently, the non-PSMA producing human prostate cancer cell line was infected with a lentiviral vector carrying the PSMA gene. This cell line produces the same order of magnitude amounts of PSMA as the human prostate cancer cell line LNCaP, figure 2. This line can be used to compare activation and efficacy of PSMA prodrugs in a matched PSMA positive and negative cell line.

In the previous progress report, we described our initial efforts to target the NAALADase activity of PSMA. After screening a series of alpha-linked dipeptides, table 1, to determine preferred PSMA substrates, a series of dipeptide methotrexate analogs were synthesized and screened for PSMA activation and non-specific cytotoxicity to PSMA-negative prostate cancer cells, table 2. From a large series of dipeptide analogs, only one prodrug, APA-Asp-Glu, was found to be hydrolyzed by PSMA. Hydrolysis of APA-Asp-Glu produces the methotrexate analog APA-Asp that is approximately 100 times less cytotoxic than methotrexate (please note for peptide sequences throughout progress report a hyphen designates alpha-linkage and star designates gamma-linkage of amino acids). In addition, the prodrug, APA-Asp-Glu, was found to be a more potent cytotoxin than the activated APA-Asp. These studies demonstrate that the addition of the APA molecule to the amino terminus of the dipeptides markedly restricts hydrolysis by PSMA. At the same time, the addition of a single amino acid in most cases did not result in inactivation.

On the basis of these studies, it became clear that targeting the NAALADase activity of PSMA using alpha-linked acidic dipeptides may not be the best strategy. Modification of the dipeptides results in marked decrease in hydrolysis by PSMA and the dipeptides may not adequately neutralize the cytotoxicity of the TG analog.

In the original proposal, targeting the NAALADase activity of PSMA was proposed because this activity appeared to be unique to PSMA. Subsequent to the original submission of this proposal, new data has emerged demonstrating that human prostate PSMA, rat brain NAALADase and pig jejunal folate hydrolase have a high degree of sequence identity. In addition, all three enzymes possess both folate hydrolase and NAALADase activity. Therefore, targeting the NAALADase activity of PSMA would lend no additional specificity

A second approach to targeting PSMA is to design prodrugs that can be activated by the pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase) activity PSMA. Previously it has been demonstrated that PSMA is able to progressively hydrolyze γ-glutamyl linkages of both poly-gamma glutamated folates and poly-gamma glutamated methotrexate analogs with varying length glutamate chains. These polyglutamated analogs can also be readily hydrolyzed by gamma glutamyl hydrolase, a lysosomal enzyme. Gamma glutamyl hydrolase is also secreted by hepatocytes and by a variety of tumor cell types. Therefore, an ideal PSMA-prodrug would be specifically hydrolyzed by PSMA with minimal to no hydrolysis by the ubiquitous gamma glutamyl hydrolase.

The first step toward accomplishing this goal was to more clearly define substrate specific for the pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase) activity of PSMA. Therefore, a series of γ-linked methotrexate analogs were synthesized and assayed for hydrolysis by PSMA. Initially, dipeptide gamma-linked

analogs were screened, table 3. These dipeptide analogs were poorly hydrolyzed by PSMA and were non-specific cytotoxins. Subsequently, a series longer chain gamma-linked analogs were synthesized and characterized. The analogs APA-$(Glu)_4$*Asp and APA-$(Glu)_4$*Gln, in which the terminal glutamate is replaced by γ-linked Asp or Gln were both hydrolyzed by PSMA, table 3. These analogs, however were less efficient substrates when compared to APA-$(Glu)_5$, the polyglutamated methotrexate analog with similar γ-glutamyl chain length. These analogs were also tested for cytotoxicity against TSU cells, table 3. The gamma linked analogs APA-$(Glu)_5$, APA-$(Glu)_4$*Asp, APA-$(Glu)_4$*Gln were cytotoxic and each produced >95% loss of clonogenic ability at 10 μM, figure 2. APA-$(Glu)_5$ was the most potent analog at lower doses with > 90 % inhibition at 1 μM and ~40% inhibition at 0.1 μM, table 3.

Previously, the hydrolysis of polyglutamated methotrexate [APA-$(Glu)_5$] by γ-glutamyl hydrolase (GGH) has been demonstrated. GGH is a cysteine protease found predominantly in the lysosomes of most cell types. However, GGH is also secreted by a variety of transformed cell lines as well as some normal cell types, particularly hepatocytes. GGH activity is also present within human serum. Because these prodrugs will be administered systemically via the blood, it is important to determine their stability in sera, particularly because GGH activity is also know to be present within the serum. Therefore, the APA-$(Glu)_5$ (i.e. polyglutamated methotrexate), APA-$(Glu)_4$-Asp, APA-$(Glu)_4$-Gln were incubated in both mouse and human plasma and hydrolysis determined by HPLC analysis at various time points, table 4. For each of these analogs, approximately 50% conversion of the starting material to methotrexate was seen after 18 h incubation in human plasma, table 4. In addition, all of the intermediate species were detectable, consistent with sequential hydrolysis by the exopeptidase activity of GGH present in the serum. Similar results were seen in mouse plasma, with the exception of less total conversion to methotrexate when compared to human plasma, table 4. The addition of p-hydroxymercuribenzoate, a non-specific inhibitor of cysteine proteases resulted in complete inhibition of hydrolysis, again consistent with inhibition of GGH. In contrast, the alpha-linked APA-Asp-Glu analog was completely stable to hydrolysis in serum (data not shown).

These studies have delineated the limited range of a-linked substrates that can be hydrolyzed by PSMA. The only a-linked methotrexate substrate that was hydrolyzed by PSMA (i.e. APA-Asp-Glu), however, was stable to hydrolysis by GGH present in the serum. These studies have also demonstrated the enhanced ability of PSMA to hydrolyze a variety of g-linked substrates. These g-linked substrates, however, are not specific for PSMA but also hydrolyzed by GGH. The ideal substrate, therefore, would take advantage of the dual ability of PSMA to hydrolyze certain alpha and gamma linkages between aspartyl and glutamyl residues, figure 2. Thus, the ideal substrate should incorporate the specificity of the a-linkage with the enhanced efficiency of the g-linkage. The longer length, negatively charged, substrates would serve two additional purposes: first, they help to make the highly lipophilic more toxins like TG analogs more water soluble; second, the highly charged prodrug will be less likely to cross the plasma membrane, further limiting non-specific cytotoxicity, figure 2.

Using this rationale, additional substrates were synthesized in which a PSMA-hydrolyzable a-linked dipeptide "cap" is introduced that is not a substrate for GGH in order to produce more specific PSMA substrates, table 5. One of these substrates APA-Glu*Glu*Glu*Asp-Glu was a less efficient PSMA substrate but showed enhanced stability in human serum. A second substrate APA-Glu*Glu*Glu*Asp-Gln was a poor substrate for PSMA, although it had even more enhanced stability in serum. Finally, a substrate containing two a-linkages and two g-linkages, APA-Asp-Glu*Glu*Asp-Glu was a better PSMA substrate and was completely stable to hydrolysis in human and mouse plasma, table 1. These combination alpha- and gamma-linked PSMA substrates possess the best combination of efficiency and specificity and these substrates will therefore be used to create prodrugs described below.

In a final set of experiments, these alpha- and gamma-linked analogs, APA-Glu*Glu*Glu*Asp-Glu and APA-Asp-Glu*Glu*Asp-Glu were administered to nude mice to determine toxicity in whole animals. Previously we had established that a dose of 20 mg/kg methotrexate administered subcutaneously to mice was 100% toxic within 10 days post therapy. Therefore, equitoxic doses of the two methotrexate prodrugs were administered to groups of animals. The APA-Glu*Glu*Glu*Asp-Glu was 100% toxic to mice within the same

time frame (n=5), while the APA-Asp-Glu*Glu*Asp-Glu produced no demonstrable toxicity (i.e. no deaths and no weight loss). Further studies are required to fully determine the stability and in vivo metabolism of this prodrug, however, these studies suggest that the APA-Asp-Glu*Glu*Asp-Glu peptide may be a stable drug carrier that is not appreciably hydrolyzed by normal tissues or serum.

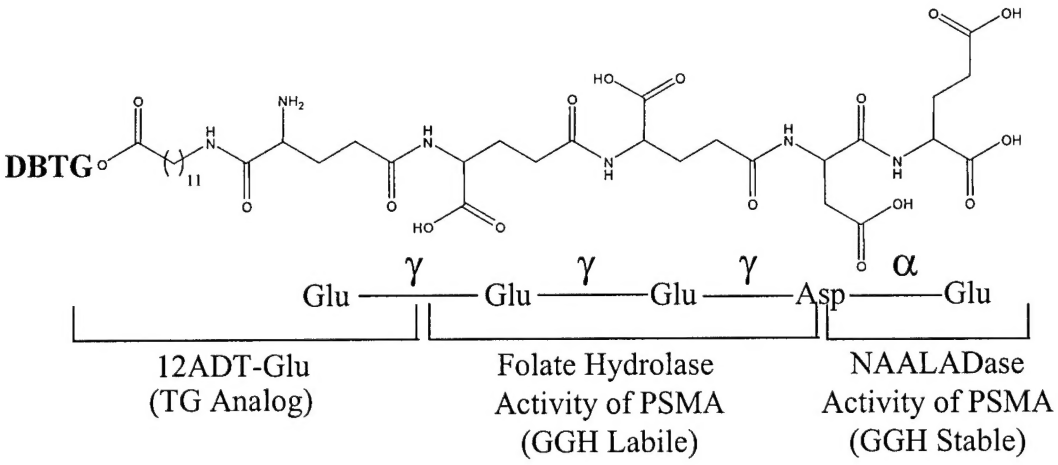


Figure 3. Example of PSMA prodrug containing α-and γ-linked amino acids and TG analog 12ADT-Glu.

Progress with Thapsigargin Analogs

Thapsigargin (TG) is a sesquiterpene γ-lactone isolated from the root of the umbelliferous plant, *Thapsia garganica*. TG has been shown to increase intracellular Ca^{2+} and induce programmed cell death in prostate cancer cell lines as well as a host of other normal and malignant cell types. More recent studies have shown that TG inhibits the sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+}-ATPase (SERCA) pump with an IC_{50} value of 30 nM. In work supported by the DOD, we demonstrated that in cells treated with TG a bi-phasic changes in intracellular free calcium is observed. After an initial increase from baseline values of 20-40 nM to values of 200-400 nM, induced by the emptying of the endoplasmic reticulum calcium pool, intracellular calcium return to baseline values within 6 to 18 hours. This decrease is mediated by activation of the calmodulin-dependent calcium pump of the plasma membrane since cells micro-injected with a calmodulin inhibiting peptide maintained an elevated calcium. This first rise can be attenuated by intracellular buffers such as BAPTA or calcium binding protein calbindin. In all dying cells we have observed a sustained second elevation of intracellular free calcium from a baseline of 20 -40 nM to 10- 50 μM. This rise to μmolar values precedes the morphological changes associated with apoptosis in both prostate and breast cancer cells. This second rise is asynchronous within the cell population but ultimately occurred in every dying cells. The proportion of cells showing a second increased per unit of time correlates with the number of cells showing DNA fragmentation and the proportion of cells showing loss of viability when measured by clonogenic assay. These results demonstrate the critical role of sustained elevations of intracellular Ca^{2+} in the programmed cell death induced by TG..

TG induces programmed cell death of all rapidly proliferating prostate cancer cell lines. Unlike standard antiproliferative agents such as 5-FU or doxorubicin, TG can also induce apoptosis in non-proliferating, G_0 arrested prostate cancer cells. To demonstrate this, primary cultures of human prostate cancer cells were made. These cultures initially grow exponentially. During this exponential phase treatment with equal concentrations of either the cell proliferation-dependent chemotherapeutic agents 5-FrdU or doxorubicin or thapsigargin resulted in sterilization of culture dishes. In contrast, these cells were shown to go out of cycle and enter the proliferatively quiescent G_0 state after an initial proliferation period of approximately 10 days. When these

stationary cultures were exposed for 1 week to effective doses (i.e.100 nM) of doxorubicin or 5-FrdU, there was no activation of PCD either morphologically by videomicroscopic evaluation or quantitatively by DNA fragmentation . In contrast, exposure of the stationary cultures to 100 nM TG resulted in morphologic changes within 24 to 48 hours, with fragmentation into apoptotic bodies starting by day 4.

On the basis of these preclinical studies, it would appear that TG represents an excellent choice for treatment of prostate cancer because of its ability to kill these cells in a proliferation-independent manner. Unfortunately, while TG is highly effective in inducing the proliferation independent programmed cell death of androgen independent prostate cancer cells, it is not cell type specific and is sparingly water soluble due to its high lipophilicity. In order to target TG's cytotoxicity specifically to prostate cancer cells systemically, TG must be chemically modified to produce an analog that can be coupled to a water-soluble peptide carrier. This modification involves the introduction of a primary amine containing side chain into the TG molecule that can be coupled via a peptide bond to the carboxyl group of the C-terminal amino acid. In this way, TG can be targeted specifically to metastatic deposits of androgen independent prostate cancer producing enzymatically active PSMA.

Our collaborator on this project, Dr. S. Brogger Christensen, professor of medicinal chemistry at the Royal Danish School of Pharmacy, Copenhagen, Denmark, originally isolated and chemically characterized TG. Based on a model of the TG binding site within the SERCA pump it was determined that modifications of the TG molecule could possibly be made in the side chain in the 8-position without adversely effecting SERCA pump inhibitory activity. Using this rationale a series of TG analogs (i.e. ~25) modified in the 8-position with primary amine containing side chains were synthesized. These analogs were characterized for their ability to inhibit the SERCA pump and elevate intracellular calcium. In addition, these analogs were assayed for cytotoxic activity against androgen independent human prostate cancer cells in vitro. From these studies we had identified a lead TG analog, 8-aminophenylproprionyl TG (APT) that maintained ability to inhibit the ATPase and elevates intracellular calcium. This analog, however, was approximately a 10-fold less potent cytotoxin than TG.

Because PSMA is an exopeptidase, hydrolytic processing of any prodrug will result in an end product consisting of a cytotoxin coupled to an acidic amino acid, most likely glutamate. Therefore, while a TG analog can be potentially targeted using this prodrug approach, preferred analogs would be those that incorporate glutamic acid or some other dicarboxylic acid into their structure and still maintain their cytotoxicity. Analogs of APT were made in which a series of amino acids were coupled to the free amine of APT. The best of this group of analogs was 40-fold less potent than TG and 4-fold less potent than APT against prostate cancer cell lines.

On the basis of these results, a series of additional primary amine containing TG analogs coupled to amino acids were synthesized and assayed for activity against human prostate cancer cell lines. These analogs contained long hydrocarbon side chains and ended in a primary amine . The analog was then coupled to a series of amino acids. Incorporation of an amino acid into the structure of TG produces a more water-soluble species that would be less likely to cross the plasma membrane. In addition, incorporation of an amino acid could potentially disrupt binding to the SERCA pump. Therefore, long hydrocarbon linkers were introduced to make the analogs more hydrophobic and to also move the amino acid tail away from the TG binding site within the SERCA pump. One of theses analogs, containing a 12-amino dodecanoate side chain (12ADT) was found to have an IC_{50} value against TSU cells of ~ 500 nM. When this long chain analog was coupled to the amino acid l-leucine to produce L-12ADT the IC_{50} value shifted to 75 nM. The 12ADT was next coupled to the gamma carboxyl of glutamic acid to produce E-12ADT. This compound has an IC_{50} value against TSU cells of ~ 200 nM. Similarly, the 12ADT was coupled to the beta-carboxyl of aspartate to produce D-12ADT. This analog was also a potent cytotoxin with similar IC_{50} value against TSU cells.

Figure 1. Schematic of Methotrexate structure

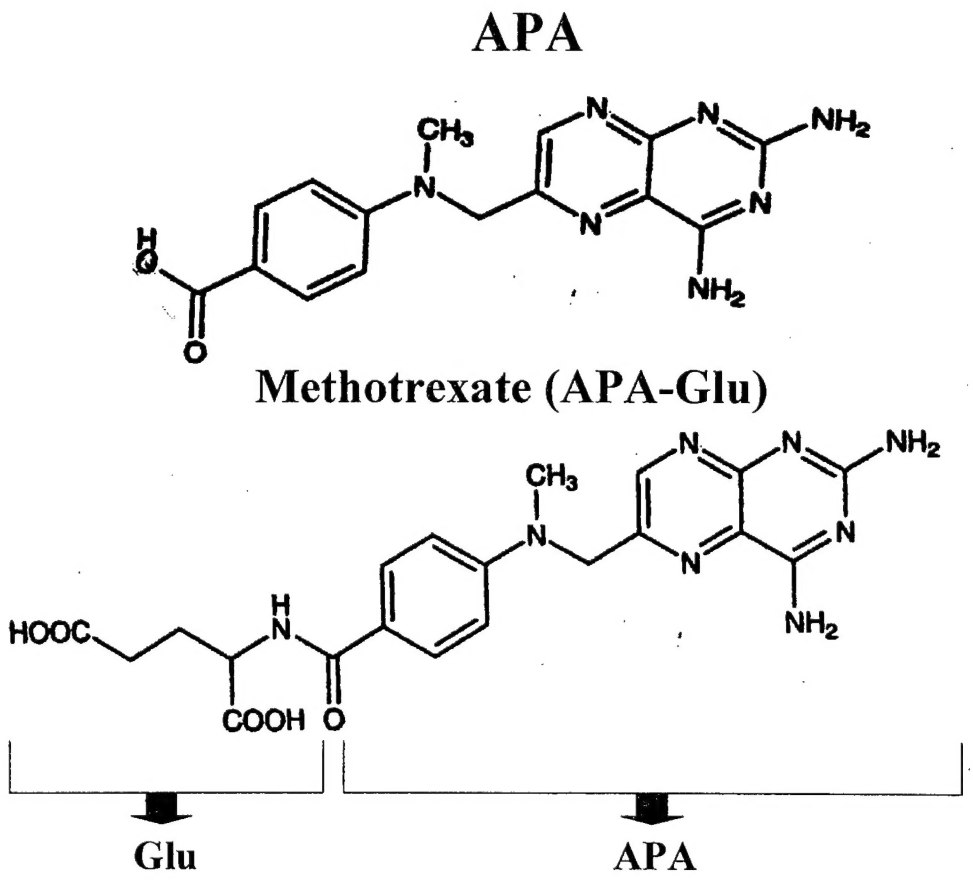
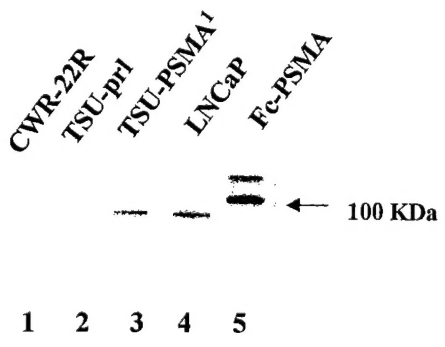


Figure 2. Western Blot analysis of PSMA production by prostate cancer cell lines, Lentiviral infected TSU cells and purified Fc-PSMA.



Immunoblot analysis of the cell lines used to test the activity of Methotrexate Analogs. An equivalent amount of protein (500μg) from TSU-prl cells, LNCaP cells and CWR-22R cells was analyzed along with 125μg of TSU-PSMA and 0.25μg of Fc-PSMA. Proteins were separated using 12% SDS-PAGE and transferred onto a Hybond ECL™ nitrocellulose membrane and probed with Y-PSMA1 antibody.

[1]TSU-PSMA cells = TSU-prl cells transfected with a lenti-viral vector encoding PSMA gene

Table 1. Peptides incubated with PSMA for 1hr and then assayed for glu release.

Peptide	% hydrolysis
D-Glu-D-Glu-OH	0%
Gln-Gln-OH	0%
Glu-Glu-Asp-OH	0%
Leu-Glu-OH	0%
Glu-Glu-Glu-OH	2%
aGlu-aGlu-Glu-OH	6%
N-Acetyl-Asp-Glu	41%
Gln-Glu-OH	53%
Glu-Asp-OH	57%
Glu-Glu-BNA	64%
Glu-BNA	73%
Glu-Glu-OH	81%
gGlu-gGlu-Glu-OH	85%

Peptides incubated with PSMA for 1hr and then assayed for asp release.

Peptide	% hydrolysis
Asp-Asp-Asp-OH	22%
Asp-Asp-Asp-Asp-OH	24%
Asn-Glu-OH	57%
Asp-Glu-OH	78%
Asp-Gln-OH	83%
Glu-Asp-OH	87%
Glu-Glu-OH	87%
Asp-Asp-OH	89%

Table 2. α-linked methotrexate analogs hydrolyzed to methotrexate after being incubated with PSMA for 24hrs and 48hrs.

Methotrexate Analog	% hydrolysis (24hrs)[1]	% hydrolysis (48hrs)	% Inhibition of Clonal Survival (1μM)
APA-Asp-OH	0.0%	0.0%	61%
APA-Glu-OH (Methotrexate)	0.0%	0.0%	97%
APA-Asp-Glu-OH	70.0%[2]	99.0%[2]	63%
APA-Glu-Glu-OH	0.0%	20.0%	61%
APA-Glu-Gln-OH	0.0%	0.0%	0%
APA-Asn-Glu-OH	0.0%	0.0%	0%
APA-Glu-Glu-Glu-OH	0.0%	0.0%	32%
APA-Glu-Asp-OH	0.0%	0.0%	0%
APA-Leu-Glu-OH	0.0%	0.0%	12%

[1]% hydrolysis determined using HPLC analysis and defined by ratio of the area of methotrexate peak divided by total area (starting material peak + methotrexate peak + intermediate peaks if present)
[2]Hydrolysis to APA-Asp
Abbreviations: APA, 4-N[N-(2,4Diamino-6-pteridinyl-methyl)-N-methylamino-benzoyl]; α-linked indicates carboxyl position used to couple to next amino acid in sequence.

Table 3. γ linked methotrexate analogs hydrolyzed to methotrexate after being incubated with PSMA for 24hrs and 48hrs.

Methotrexate Analog	% hydrolysis (24hrs)[1]	% hydrolysis (48hrs)	% Inhibition of Clonal Survival (1μM)
APA-Glu-γGABA-OH	0.0%	0.0%	96 ± 1
APA-Glu-γDGlu-OH	0.0%	0.0%	4 ± 25
APA-Glu-γAsp-OH	38.3%	84.6%	37 ±5
APA-Glu-γGln-OH	48.2%		93 ± 1
APA-Glu-γAsp-βGlu-OH	37.8%[2]	85.5%[2]	47 ± 13
APA-Glu-γGlu-γGlu-γGlu-γAsp-OH	40.9%	77.7%	65 ± 5
APA-Glu-γGlu-γGlu-γGlu-γGln-OH	6.1%	15.5%	63 ± 7
APA-Glu-γGlu-γGlu-γGlu-γGlu-OH	96.1%	100.0%	95 ± 2

Table 4. α and γ linked methotrexate analogs hydrolyzed to methotrexate after being incubated with PSMA for 24hrs and 48hrs.

Methotrexate Analog	% hydrolysis (24hrs)[1]	% hydrolysis (48hrs)	% Inhibition of Clonal Survival (1μM)
APA-Glu-γGlu-αGlu-γGlu-γGlu-OH	1.3%	5.1%	17 ± 6
APA-Glu-γGlu-γGlu-αAsp-γGlu-OH	29%	64%	37 ± 10
APA-Glu-γGlu-γGlu-αAsp-γGln-OH	4.6%	10%	15 ± 5
APA-αAsp-γGlu-γGlu-αAsp-γGlu-OH	36%[3]	77%[3]	3 ± 2

[1]% hydrolysis determined using HPLC analysis and defined by ratio of the area of methotrexate peak divided by total area (starting material peak + methotrexate peak + intermediate peaks if present)
[2]Hydrolysis to APA-Glu-γAsp only with no production of methotrexate observed
[3]Hydrolysis to APA-Asp
Abbreviations: APA, 4-N[N-(2,4Diamino-6-pteridinyl-methyl)-N-methylamino-benzoyl];
α,β,γ, indicates carboxyl position used to couple to next amino acid in sequence.

Table 5. Stability of PSMA Substrates in plasma.

	18h Incubation Human Plasma		18h Incubation Mouse Plasma
Methotrexate Analog	**% APA-Glu or Asp[1]**	**% Prodrug Remaining[2]**	**% APA-Glu or Asp[3]**
APA-Glu-γGlu-γGlu-γGlu-γAsp-OH	9	20	72
APA-Glu-γGlu-γGlu-γGlu-γGln-OH	6	26	62
APA-Glu-γGlu-γGlu-γGlu-γGlu-OH	11	11	78
APA-Glu-γGlu-γGlu-αAsp-γGlu-OH	23	57	5
APA-Glu-γGlu-γGlu-αAsp-γGln-OH	13	76	ND
APA-αAsp-γGlu-γGlu-αAsp-γGlu-OH	0	100	2

[1]Denotes %t of starting material completely hydrolyzed to APA-Asp or APA-Glu
[2]Peak area of starting material/total peak area (starting material, intermediate peaks and product)
[3]No intermediate peaks seen in mouse plasma, only starting material and APA-Asp or APA-Glu

KEY RESEARCH ACCOMPLISHMENTS:

- Developed assays to screen a number of di and tri-peptides to define PSMA substrate specificity.
- Synthesized and screened alpha-linked dipeptide analogs of methotrexate for PSMA specific hydrolysis.
- Synthesized and characterized a series of gamma-linked analogs di and tripeptide analogs of methotrexate for PSMA hydrolysis and hydrolysis by gamma glutamyl hydrolase.
- Synthesized and characterized a series of longer chain gamma-linked analogs of methotrexate and characterized PSMA hydrolysis, cytotoxicity, and stability in conditioned media and human and mouse plasma.
- Synthesized and characterized a series of methotrexate analogs containing both alpha and gamma-linkages and characterized PSMA hydrolysis, cytotoxicity, and stability in conditioned media and human and mouse plasma.
- Tested several analogs for toxicity in nude mice.
- Infected a non-PSMA producing human prostate cancer cell line, TSU, with a lentiviral vector containing the PSMA gene, to produce a line that expresses high levels of enzymatically active PSMA.
- Synthesized and screened a series of TG analogs consisting of hydrocarbon linker coupled to amino acids
- Identified gamma-glutamyl 12-aminododecanoyl TG analog that can be coupled to PSMA specific peptide substrate.

REPORTABLE OUTCOMES:

Presentations:

"PSA and PSMA Prodrug Therapies", New Diagnostic and Therapeutic Perspectives in Prostate Cancer, The Catholic University of Louvain, Brussels, Belgium, 1999.

"Hydrolysis of alpha and gamma linked polypeptide analogs of methotrexate by prostate-specific membrane antigen (PSMA)". Poster Presentation at American Association of Cancer Research (AACR) meeting, New Orleans, LA, 2001.

Manuscripts and Abstracts:

1. Lapidus RG, Tiffany CW, Isaacs JT, Slusher BS. Prostate-specific membrane antigen (PSMA) enzyme activity is elevated in prostate cancer cells. Prostate 45:350-354, 2000.
2. Tombal B, Weeraratna AT, Denmeade SR, Isaacs JT. Thapsigargin Induces a Calmodulin/ Calcineurin-dependent Apoptotic Cascade Responsible for the Death of Prostatic Cancer Cells. Prostate, 43:303-317, 2000.
3. Gala J-L, Loric S, Guiot Y, Denmeade SR, et al. Diagnostic and prognostic value of prostate specific membrane antigen in transitional cell carcinoma of the bladder. Clin. Cancer Res., 6:4049-4054, 2000.
4. Gady AM, Rosen DM, Denmeade SR. Hydrolysis of methotrexate analogs by prostate-specific membrane antigen (PSMA). Proc. Am. Assoc. Cancer Res. 42:230, 2001
5. Gady AM, Rosen DM, Denmeade SR. Hydrolysis of alpha and gamma linked polypeptide analogs of methotrexate by prostate-specific membrane antigen (PSMA). In preparation, 2001.

Patent application:

"Prodrug activation by prostate-specific membrane antigen", Inventors S. Denmeade and J. Isaacs (PI) submitted May 2000.

Employment:

1. Post-doctoral fellow, Dr. Samuel Denmeade, applied for and received faculty position as Assistant Professor, The Johns Hopkins Oncology Center in July 1998.
2. Dr. Saeed Khan, a medicinal chemist who joined our group as a Research Assistant and applied for and received faculty position as Assistant Professor, The Johns Hopkins Oncology Center, July 2000.
3. Graduate Student-Carsten Jakobsen has joined the laboratory of Dr. Soeren Christensen and will work full-time on design and synthesis of TG analogs.

CONCLUSIONS:

To date the studies with the methotrexate analogs have helped define the substrate specificity of PSMA. We have determined that the initial stated goal of targeting the NAALADase activity of PSMA using dipeptide carriers may not represent the best approach to achieving PSMA targetable drugs. Through additional studies aimed at targeting the folate hydrolase activity of PSMA, other potential PSMA specific substrates have been identified. These substrates are hydrolyzed by PSMA but also appear to be hydrolyzed by gamma glutamyl hydrolases present in human sera. The best substrates appear to be those that combine both alpha-and gamma linkages into the sequence. We have synthesized several such analogs and defined the sequence Asp-Glu*Glu*Asp-Glu as the best putative carrier to date due to its hydrolysis by PSMA and stability in human sera. While methotrexate incorporation has been useful in the biochemical analyses of substrate hydrolysis due to its strong absorbance at 340 nm, this agent is not an ideal candidate for prodrug development due to PSMA-hydrolysis independent uptake of methotrexate analogs containing long peptide side chains.

Due to the presence of these folate transport mechanisms, other cytotoxic compounds that are not recognized by the folate carriers would represent a better choice for PSMA prodrug development. In the original proposal, we outlined the characteristics of TG that would make it an excellent candidate for prodrug development. Because PSMA is an exopeptidase, the end product of PSMA hydrolysis will be a compound containing an acidic amino acid such as aspartate or glutamate. Therefore, the preferred TG analogs will maintain activity when coupled to such amino acids. We have identified an analog of TG, E-12ADT, that maintains cytotoxicity after incorporation of a glutamic acid residue into the structure.

In future studies we plan to synthesize prodrugs consisting of the cytotoxic TG analog 12-ADT coupled to the Asp-Glu*Glu*Asp-Glu substrate. This prodrug will be tested in vitro and in vivo against PSMA-producing cell lines. In additional studies, we will continue to optimize the PSMA peptide substrate in terms of optimal sequence and optimal length. Based on these studies, a series of TG prodrugs will be tested and a lead TG prodrug will be defined. We will also consider coupling other cytotoxic agents to this PSMA prodrug.

This project was supported through a phase I Department of Defense Idea Development Award. In addition, a mechanism was established with this award to allow investigators to reapply for an additional two years of funding (i.e. Phase II award) after 24 months. Under this mechanism, a limited number of the originally funded projects were to receive additional support. Our group applied for this Phase II funding with Dr. Isaacs again serving as the Principal Investigator. This proposal received a sufficiently outstanding review to be evaluated as part of the second-tier integration panel. Unfortunately, at the integration panel level it was not approved for funding. We are continuing to acquire preliminary data in order to apply for additional funding for this project.

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